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(54) Title: ANTIPROTOZOAL HISTONE ACETYL TRANSFERASE INHIBITORS

(57) Abstract: Histone acetyl transferase inhibition provides a target for identifying potential antiprotozoal compounds. Histone acetyl transferase inhibitors are useful as therapeutic agents against protozoal infections. A method of identifying histone acetyl transferase inhibitors compares i) the acetylation level from a combination of a test compound with a known histone deacetylase inhibitor to ii) a basal acetylation level.

TITLE OF THE INVENTION

ANTIPROTOZOAL HISTONE ACETYL TRANSFERASE INHIBITORS

BACKGROUND OF THE INVENTION

This invention is related to a method to identify pharmaceutical antiprotozoal compositions. In particular, this invention is directed to a method to identify antiprotozoal compositions that inhibit histone acetyl transferase.

Parasitic protozoa are responsible for a wide variety of infections in man and animals. Many protozoan infections are life threatening to the host. Consequently, such infections pose significant health and economic threats. Health threats caused by protozoa include, for example, malaria and trypanosomiasis. Malaria is caused by protozoa of the genus *Plasmodium* such as *P. vivax*, *P. falciparum*, *P. ovale* and *P. malariae*. Trypanosomiasis includes Chagas disease caused by *Trypanosoma cruzi* and African sleeping sickness caused by *T. brucei*. Further, protozoa such as *Pneumocystis carinii*, *Toxoplasma gondii*, and *Cryptosporidium sp.* can cause opportunistic infections, especially in people with compromised immune systems.

Economic threats from protozoa result from the mortality of infected animals and lower production yields from sick animals. An economically important protozoan disease of domesticated animals is coccidiosis, which is caused by protozoa of the genus *Eimeria*. In poultry, the infecting protozoa include *E. tenella*, *E. acervulina*, *E. necatrix*, *E. brunetti*, *E. mitis*, *E. praecox* and *E. maxima*.

Thus, there is a great and continuing need for effective anti-protozoan compounds.

For some protozoal diseases, such as Chagas disease, there is no satisfactory treatment. Further, in other protozoal diseases, drug-resistant strains of the infecting protozoa are decreasing the effectiveness of present treatment drugs. Thus, it would be desirable to develop new and effective anti-protozoal compounds that utilize a new mechanism against protozoa – that of inhibiting histone acetyl transferase biological intracellular activity in protozoa. Accordingly, it would be desirable to develop a method of identifying such new histone acetyl transferase inhibiting anti-protozoal compounds.

It is well known that DNA has the basic structure of a double helix. Instead of being merely a dangling string, however, the DNA double helical structure is further organized by having portions of it wrapped about a core - much as thread is

wrapped about a bobbin. Histones form the core about which the DNA is wrapped. The DNA wrapped/histone structure is known as a nucleosome or chromatin. The nucleosomes can be further organized to form higher order structures.

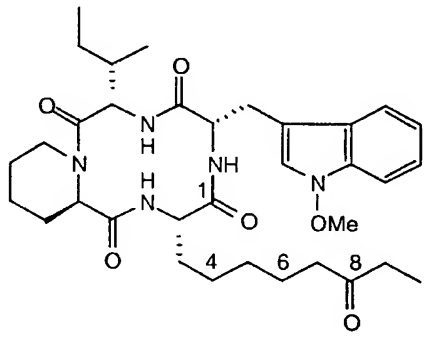
Without being bound to theory, it is believed that various biological activities depend from the geometric conformations of the participating molecules. The core histones have amino terminal regions, rich in lysine, that are ready targets for acetylation. DNA has lower affinity to acetylated histone than to deacetylated histone. As a result, the DNA is held less tightly to the acetylated histone core and the acetylated histone terminal regions are less rigidly held in relation to the DNA. Thus, the local chromatin environment is dramatically changed by acetylation, thereby affecting biological activity.

Acetylation of histone results from the action of the enzyme histone acetyl transferase ("HAT"). Substantially acetylated histone is described as being in a state of "hyperacetylation." Deacetylation of histone results from the action of the enzyme histone deacetylase ("HDA"). Histone that is substantially without acetylation is described as being in a state of "hypoacetylation."

Various compounds such as, for example, n-butyrate, trichostatin, trapoxin or apicidin can inhibit the action of HDA. The HDA inhibiting action of apicidin is described in S. Darkin-Rattray et al., *Proc. Natl. Acad. Sci. USA*, 93:13143-13147 (1996). Trapoxin is described in H. Itazaki et al., *J. Antibiotics*, 43:1524 (1990). Recently, trichostatin A and trapoxin A have been reported as reversible and irreversible inhibitors, respectively, of mammalian HDA (see e.g., Yoshida et al., *BioAssays*, 17(5):423-430 (1995)). Trichostatin A has also been reported to inhibit partially purified yeast HDA (Sanchez del Pino et al., *Biochem. J.*, 303:723-729 (1994)). Trichostatin A is an antifungal antibiotic and has been shown i) to have anti-trichomonal activity as well as cell differentiating activity in murine erythroleukemia cells, and ii) the ability to induce phenotypic reversion in *sis*-transformed fibroblast cells (see e.g., U.S. Patent No. 4,218,478; and Yoshida et al., *BioAssays*, 17(5):423-430 (1995); and references cited therein). Trapoxin A, a cyclic tetrapeptide, induces morphological reversion of v-*sis*-transformed NIH3T3 cells (Yoshida and Sugita, *Jap. J. Cancer Res.*, 83(4):324-328 (1992).

HDA inhibition as a target for cancer research is described in Saito et al., *Proc. Natl Acad. Sci. USA*, 96:4592-4597(1999); Bernardi et al., *Amino Acids* 6:315-318 (1994); and R.E. Shute et al., *J. Med. Chem.* 30:71-78 (1987). Utilizing HDA as a target for antiprotozoal agents is described in U.S. Patent Applications

09/296,834, filed April 22, 1999, and 08/716,978, filed September 20,1996. U.S. Patent No. 4,218,478 describes the use of trichostatin as an antiprotozoal agent. Antiprotozoal cyclic tetrapeptides, including the HDA inhibitor apicidin, are described in U.S. Patent No. 5,620,953, incorporated herein by reference. Apicidin [*cyclo*(N-O-methyl-L-Trp-L-Ile-D-Pip-L-2-amino-8-oxo-decanoyl)] is a broad-spectrum antiprotozoal, antifungal and antineoplastic agent isolated from the fermentation culture of Fusarium fungus. The structure of apicidin is shown below:



Nevertheless, there remains a need to develop novel antiprotozoal agents. Particularly desirable would be new antiprotozoal agents utilizing novel properties such as HAT inhibition, as well as a method to identify such HAT inhibiting agents.

SUMMARY OF THE INVENTION

This invention provides a method for identifying compounds having antiprotozoal activity. The method includes the steps of contacting an histone acetyl transferase with (i) an identifiably labeled compound that interacts with histone acetyl transferases, and (ii) a test compound or composition; and quantitating the inhibition of the interaction of the labeled compound, wherein that inhibition is induced by the test compound or composition.

In another method, this invention identifies compounds having histone acetyl transferase inhibiting activity by the steps of i) determining a basal histone acetylation level in a histone/histone acetyl transferase/histone deacetylase system; ii) determining a reference histone hyperacetylation level in a histone/histone acetyl transferase/histone deacetylase/histone deacetylase inhibitor system; iii) determining a

test histone acetylation level in a histone/histone acetyl transferase/histone deacetylase/histone deacetylase inhibitor/test composition system; and iv) comparing the test level to the basal and reference levels.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a is a schematic diagram of the action of uninhibited HAT and uninhibited HDA on the acetylation of histone.

Fig. 1b is a schematic diagram of the action of inhibited HDA and uninhibited HAT on the acetylation of histone.

Fig. 1c is a schematic diagram of the action of inhibited HDA and inhibited HAT on the acetylation of histone.

DETAILED DESCRIPTION OF THE INVENTION

As described above, the action of HDA and HAT together control the net level of acetylation of histones. Inhibition of the action of HDA (for example, by the action of apicidin) results in the accumulation of hyperacetylated histones from the unopposed action of HAT. Such hyperacetylation accumulation can be measured. For example, an ELISA (Enzyme-linked immunosorbent assay) of apicidin treated HeLa cells can directly quantify hyperacetylated histone buildup. The ELISA can utilize antibodies such as, for example, one raised against an acetylated peptide which has a sequence identical to the amino terminus of human histone H3 or H4. Such ELISA serves as a control experiment that provides a baseline against which test compounds can be screened for each compound's effectiveness at inhibiting the action of HAT. An inhibition of the action of HAT would result in a lessening of the accumulation of hyperacetylated histones. That is, the inhibition of the action of HDA in the baseline control is counterbalanced by any inhibition of the action of HAT provided by the test candidate.

In one aspect the present invention provides a method for identifying compounds having HAT inhibition activity by adding a test compound to the above-described control experiment. After a specific duration of time, the amount of hyperacetylation is compared to the control. The lower the build-up of hyperacetylated histone, the more effective is the inhibiting action of the test compound to the action of HAT.

Referring to Fig. 1a, the basal condition of a histone/HAT/HDA system is shown in which there is an equilibrium level of acetylated histone on the

right and deacetylated histone on the left as a result of the actions of HAT and HDA. This equilibrium level of acetylation can be measured as a basal level. By the initial addition of an amount of an HDA inhibitor, referring to Fig. 1b, an augmented concentration of acetylated histone is caused by the decreased action of the inhibited HDA. Thus, in the histone/HAT/HDA/HDA inhibitor system a condition of hyperacetylation exists. This hyperacetylation level of acetylation can be measured as a reference or control level of acetylation (hyperacetylation). Referring to Fig. 1c, when an active test candidate (one that effectively inhibits HAT) is added together with the HDA inhibitor to provide a histone/HAT/HDA/HDA inhibitor/test compound system, both HDA and HAT are inhibited and the acetylation condition resembles the basal equilibrium.

Therefore, an ineffective HAT inhibiting test candidate would lead to an acetylation condition closer to Fig. 1b than to Fig. 1a (thus resembling hyperacetylation or the reference level of acetylation), while an effective HAT inhibiting test candidate would lead to an acetylation condition closer to Fig. 1a (thus resembling the basal level of acetylation). Accordingly, the present invention generally provides for i) measuring a basal acetylation condition, ii) measuring an inhibited histone deacetylase reference condition, iii) measuring a test acetylation condition of histone deacetylase inhibition in combination with histone acetyl transferase inhibition from a HAT inhibition candidate, and iv) comparing the test measurement to the other two (basal and reference) measurements. An active HAT inhibiting candidate will provide a measurement substantially similar to the basal condition.

Thus, in one aspect the present invention identifies compounds having histone acetyl transferase inhibiting activity by a method that includes the steps of i) determining a basal histone acetylation level in a histone/histone acetyl transferase/histone deacetylase system; ii) determining a reference histone hyperacetylation level in a histone/histone acetyl transferase/histone deacetylase/histone deacetylase inhibitor system; iii) determining a test histone acetylation level in a histone/histone acetyl transferase/histone deacetylase/histone deacetylase inhibitor/test composition system; and iv) comparing the test level to the basal and reference levels.

Techniques to determine histone acetylation levels are well known in the art. Any convenient technique may be used such as, for example, measuring the optical density of ELISA plates or measuring the radioactivity level of tritium marked

reagents. One in the art readily understands the applicability of particular techniques in given situations.

Accordingly, a method of this invention includes the steps of

A) contacting histone acetyl transferase with (i) an identifiably labeled compound that interacts with histone acetyl transferases; and (ii) a test compound or composition; and

B) quantitating the labeled compound to determine a level of histone acetyl transferase that is induced by the test compound.

That is, the test compound or composition, by affecting the action of HAT, affects the quantifiable amount of the labeled compound. Thus, quantification of the labeled compound serves as a diagnostic surrogate for determining any inhibiting of HAT by the test compound.

One embodiment of the present invention provides a method for identifying compounds having HAT inhibition activity comprising the steps of:

a) contacting with histone i) a known amount of histone deacetylase, ii) a known amount of histone acetyl transferase, and iii) a known amount of a labeled compound that interacts with histone acetyl transferase;

b) measuring the level of histone acetylation and setting the measured level as a baseline (zero, basal or background) level;

c) contacting with histone i) a known amount of histone deacetylase, ii) a known amount of histone acetyl transferase, iii) a known amount of a labeled compound that interacts with histone acetyl transferase, and iv) a known amount of an histone deacetylase inhibitor, wherein (c)(i), (c)(ii), and (c)(iii) are substantially in the same proportions to each other as (a)(i), (a)(ii), and (a)(iii) above respectively;

d) quantitating the inhibition of the HDA by measuring the level of histone acetylation (hyperacetylation) after a set duration of time and setting the measured level as a hyperacetylation or reference level;

e) contacting with histone i) a known amount of histone deacetylase, ii) a known amount of histone acetyl transferase, iii) a known amount of a labeled compound that interacts with histone acetyl transferase, iv) a known amount of an histone deacetylase inhibitor, and v) a known amount of a test compound, wherein (e)(i), (e)(ii), (e)(iii), and (e)(iv) are substantially in the same proportions to each other as (c)(i), (c)(ii), (c)(iii), and (c)(iv) above respectively;

f) quantitating the affect of the test compound by measuring the level of histone acetylation after a set duration of time substantially the same duration as that of (d) and setting the measured level as a test level; and

g) quantitating the HAT inhibiting effect of the test compound by comparing the results of (f) with (d) and (b).

In still another embodiment, the present invention provides a method for identifying compounds having HAT inhibition activity comprising the steps of:

aa) contacting with histone i) a known amount of histone deacetylase, ii) a known amount of histone acetyl transferase, iii) a known amount of a labeled compound that interacts with histone acetyl transferase, and iv) a known amount of an histone deacetylase inhibitor,

bb) quantitating the inhibition of the HDA by measuring the level of histone acetylation (hyperacetylation) after a set duration of time and setting the measured level as a reference level;

cc) contacting with histone i) a known amount of histone deacetylase, ii) a known amount of histone acetyl transferase, iii) a known amount of a labeled compound that interacts with histone acetyl transferase, iv) a known amount of an histone deacetylase inhibitor, and v) a known amount of a test compound, wherein (cc)(i), (cc)(ii), (cc)(iii), and (cc)(iv) are substantially in the same proportions to each other as (aa)(i), (aa)(ii), (aa)(iii), and (aa)(iv) above respectively;

dd) quantitating the affect of the test compound by measuring the level of histone acetylation after a set duration of time substantially the same duration as that of (bb) and setting the measured level as a test level; and

ee) quantitating the HAT inhibiting effect of the test compound by comparing the results of (dd) with (bb).

Another method of this invention includes the steps of:

A) contacting histone with i) a histone acetyl transferase, (ii) a histone deacetylase, (iii) a histone deacetylase inhibitor, (iv) a test compound or composition, and (iii) an antibody that interacts with acetylated histone; and

B) quantitating the antibody to determine a level of acetylated histone induced by the test compound.

That is, by affecting the action of HAT, the test compound or composition affects the level of histone acetylation - which can be monitored by quantifying the antibody. Thus, quantification of the antibody serves as a diagnostic surrogate for determining any inhibiting of HAT by the test compound.

In an embodiment, the present invention provides a method for identifying compounds having HAT inhibition activity comprising the steps of:

aaa) contacting with histone i) a known amount of histone deacetylase, ii) a known amount of histone acetyl transferase, and iii) a known amount of an antibody that interacts with acetylated histone;

bbb) measuring the level of histone acetylation and setting the measured level as a baseline (zero, basal or background) level;

ccc) contacting with histone i) a known amount of histone deacetylase, ii) a known amount of histone acetyl transferase, iii) a known amount of an antibody that interacts with acetylated histone, and iv) a known amount of an histone deacetylase inhibitor, wherein (ccc)(i), (ccc)(ii), and (ccc)(iii) are substantially in the same proportions to each other as (aaa)(i), (aaa)(ii), and (aaa)(iii) above respectively;

ddd) quantitating the inhibition of the HDA by measuring the level of histone acetylation (hyperacetylation) after a set duration of time and setting the measured level as a hyperacetylation or reference level;

eee) contacting with histone i) a known amount of histone deacetylase, ii) a known amount of histone acetyl transferase, iii) a known amount of an antibody that interacts with acetylated histone, iv) a known amount of an histone deacetylase inhibitor, and v) a known amount of a test compound, wherein (eee)(i), (eee)(ii), (eee)(iii), and (eee)(iv) are substantially in the same proportions to each other as (ccc)(i), (ccc)(ii), (ccc)(iii), and (ccc)(iv) above respectively;

fff) quantitating the affect of the test compound by measuring the level of histone acetylation after a set duration of time substantially the same duration as that of (ddd) and setting the measured level as a test level; and

ggg) quantitating the HAT inhibiting effect of the test compound by comparing the results of (fff) with (ddd) and (bbb).

In another embodiment, the present invention provides a method for identifying compounds having HAT inhibition activity comprising the steps of:

AA) contacting with histone i) a known amount of histone deacetylase, ii) a known amount of histone acetyl transferase, iii) a known amount of an antibody that interacts with acetylated histone, and iv) a known amount of an histone deacetylase inhibitor;

BB) quantitating the inhibition of the HDA by measuring the level of histone acetylation (hyperacetylation) after a set duration of time and setting the measured level as a hyperacetylation or reference level;

CC) contacting with histone i) a known amount of histone deacetylase, ii) a known amount of histone acetyl transferase, iii) a known amount of an antibody that interacts with acetylated histone, iv) a known amount of an histone deacetylase inhibitor, and v) a known amount of a test compound, wherein (CC)(i), (CC)(ii), (CC)(iii), and (CC)(iv) are substantially in the same proportions to each other as (AA)(i), (AA)(ii), (AA)(iii), and (AA)(iv) above respectively;

DD) quantitating the affect of the test compound by measuring the level of histone acetylation after a set duration of time substantially the same duration as that of (BB) and setting the measured level as a test level; and

EE) quantitating the HAT inhibiting effect of the test compound by comparing the results of (DD) with (BB).

The amount of histone deacetylase and histone acetyl transferase above can be those amounts found in a specific cell line, as well as extracellular known amounts. That is, the enzyme amounts can be that found intracellularly. The enzyme amounts can also be that amount mixed extracellularly.

In another embodiment, i) the basal acetylation of histone is first measured by collecting subject cells, extracting their histones, placing the extractions on AUT gels, followed by quantitating by Western blots or with protein stains the amount of histone acetylation in a first measurement. ii) Second, the control amount of histone hyperacetylation is measured by dosing the same type subject cells with a control amount of a control HDA inhibitor, collecting the cells, extracting their histones, placing the extractions on AUT gels, and quantitating by Western blots or with protein stains the amount of acetylation (hyperacetylation) in a second measurement. iii) Third, under the same conditions as (ii), a known amount of a test compound is added together with the control amount of the control HDA inhibitor, and the amount of hyperacetylation of histone is quantitated by Western blot or with protein stains in a third measurement. The third measurement is compared to the first and second measurements to quantitate the inhibiting property of the test compound to HAT activity.

In another aspect, another method of this invention determines the HAT inhibiting property of a test candidate by i) adding a specific amount of an HDA inhibitor together with a lesser known amount of a test candidate to a histone/HAT/HDA system and measuring the histone acetylation level, ii) adding a specific amount of an HDA inhibitor together with an augmented known amount of a test candidate to a histone/HAT/HDA system and measuring the histone acetylation

level, and iii) comparing the measured histone acetylation levels. An active HAT inhibitor will yield a lower histone acetylation level from the addition of an augmented amount than from the addition of a lessor amount of test candidate.

Any of the above methods can be used to compare the effect of test candidates on protozoal HAT and on host HAT. It is apparent that the test candidates that more potently inhibit protozoal HAT, compared to their potency at inhibiting host HAT, are likely effective antiprotozoal compounds. Test candidates that affect protozoal and host HAT approximately the same, however, are also antiprotozoal compounds. Without being bound by theory, it is believed that the reproductive rate of protozoa being usually much greater than that rate of the host cells causes protozoa to be more susceptible to HAT inhibition. Therefore, compounds showing any substantial protozoal HAT inhibition properties are antiprotozoal compounds. It is preferred that the test compounds inhibit protozoal and host HAT about equally. It is more preferred that the test compounds inhibit protozoal HAT greater than host HAT.

In another aspect, the present invention provides a method for identifying compounds having antiprotozoal activity, by HAT inhibition, comprising the steps of:

I) determination of HAT inhibition activity:

(a) contacting an intact host or protozoal cell with a test compound and a known amount of an HDA inhibiting compound;

(b) disrupting the host or cell to obtain histones;

(c) determining the level of histone acetylation; and

(d) comparing the level of histone acetylation to (i) the level of acetylation from the action of the HDA inhibiting compound without the presence of the test compound and to (ii) the level of acetylation of the cell without any HDA inhibiting or test compound to determine if the test compound is an effective (active) HAT inhibitor.

II) determination of selective HAT inhibition by taking active candidates and testing against parasites such as *E. tenella* for anti-protozoal activity by any convenient method, such as described in D.M. Schmatz, et al., *J.Protozoology*, 33(1):109-114 (1986):

(a) in parallel experiments each having known amounts of host cells and parasite, a screen experiment adds a candidate active HAT

inhibitor composition to the host/parasite while a control experiment does not include such added composition;

(b) in the first screening decision, a candidate that shows a continuing presence of the host in comparison to the decreasing presence of the host in the control passes to the next screening decision;

(c) the viability of the host is determined after a specified amount of time and compared to the viability of the parasite after the specified amount of time;

(d) the viabilities determined are compared; a good selective candidate is one that kills the parasite and not the host.

Thus, this invention provides facile and specific assays to screen compounds as HAT inhibitors and as potential antiprotozoal drugs.

In the present invention, the HDA can be any convenient protein or mixture of proteins known in the art such as, for example, a native protein in whole cells, a purified or partially purified native enzyme, a cloned histone deacetylase or an engineered variant thereof, a crude preparation of the enzyme, or an extract containing histone deacetylase activity. The enzyme can be from a mammalian (e.g. human cervical carcinoma, HeLa cell), avian (e.g. chicken liver or erythrocyte nuclei) or protozoal (e.g. *Eimeria tenella* or *P. Berghei*) source. Preferably, a protozoal histone deacetylase is used. Fragments of histone deacetylase that retain the desired enzyme activity are also within the scope of this invention.

In the present invention, the HAT can be any convenient protein or mixture of proteins known in the art such as, for example, a native protein in whole cells, a purified or partially purified native enzyme, a cloned histone acetyl transferase or an engineered variant thereof, a crude preparation of the enzyme, or an extract containing histone acetyl transferase activity. Fragments of histone acetyl transferase that retain the desired enzyme activity are also within the scope of this invention.

In the present invention, the HDA inhibitor can be any convenient compound known in the art including those that act as substrates for the histone deacetylase enzyme, those that bind the enzyme at its active site, or those that otherwise act to alter enzyme activity by binding to an alternate site.

The test compound can be a synthetic compound, a purified preparation, crude preparation, or an initial extract of a natural product obtained from plant, microorganism, or animal sources.

One embodiment of the present method is based on test compound induced inhibition of histone acetyl transferase activity as measured by scintillation counting to determine the histone acetylation level. Compounds that inhibit histone acetyl transferase induced incorporation of ^3H acetyl CoA into histones – using any enzyme source (protozoan/mammalian/avian/etc. -- would be considered histone acetyl transferase inhibitors. The level of incorporation can also be determined using fluorography or autoradiography following SDS polyacrylamide gel electrophoresis.

In a preferred embodiment, the method of the present invention utilizes a histone acetyl transferase or an extract containing histone acetyl transferase obtained from a protozoal source, such as *Eimeria* or *Plasmodium* sp.

Another method, similar to that described above, determines the test compound's inhibiting properties directed selectively to the parasite. Compounds that inhibit parasite histone acetyl transferase induced incorporation of ^3H acetyl CoA into histones -- with little or no effect on chicken or mammalian histone acetyl transferase induced incorporation of ^3H acetyl CoA into histones -- would be considered selective or specific parasitic histone acetyl transferase inhibitors. Similarly, the level of incorporation can also be determined using fluorography or autoradiography following SDS polyacrylamide gel electrophoresis.

In a more preferred embodiment, the method of the present invention further comprises determining the IC_{50} of test compounds against host histone acetyl transferase in the enzyme inhibition assay as described above, to identify those compounds that have selectivity for parasitic histone acetyl transferase over that of a host. The assays are the same as previously described, with the histone acetyl transferase activity obtained from a host of protozoa; for example the host histone acetyl transferase may be obtained from a mammalian source, e.g. human, or an avian source, e.g. chicken.

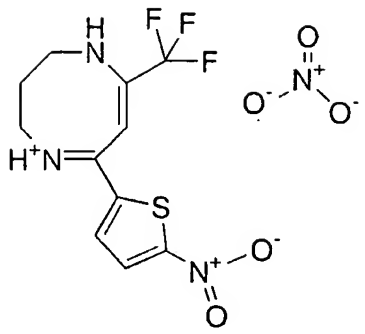
Where the enzyme inhibition or ELISA utilizes a crude preparation or an extract or a whole cell containing histone acetyl transferase, the target of the test compound may be verified by examining the level of histone acetylation. Thus, the Eimeria infected host cell containing the enzyme is treated with Apicidin (to induce hyperacetylation of histones) and the test compound. The cells are lysed and the level of histone acetylation is determined using an antibody raised against an acetylated peptide the sequence of which is identical to amino terminus of human histone H3 or H4. This is performed using an ELISA based assay. A histone acetyl transferase inhibitor will cause inhibition of apicidin induced hyperacetylation of histones. Since

this method uses intact cells treated with the test compound, this technique may also be used to identify prodrugs that may be converted to histone acetyl transferase inhibitor within the cellular environment, but may not be so identified by assay based on the enzyme itself.

Another method useful to identify inhibitors that are selective for parasitic histone acetyl transferase is the use of acid urea trion (AUT) gel electrophoresis to determine the level of acetylation of histones. Cells are treated as described in preparation for the ELISA assay but the level of hyperacetylation is determined by any convenient method such as, for example, by protein stains or on AUT gels. In the AUT system the histones of host and parasite can be separated and can be detected, following transfer to nitrocellulose, using an antibody which is specific for certain acetylated lysine residues in the amino terminal region of histone H3 or H4 as described in B.M. Turner, et al., *FEBS Lett.*, 253, 141-145 (1989). These antibodies were raised against an acetylated peptide the sequence of which is identical to the amino terminus of human histone H3 or H4. Thus compounds that inhibit the apicidin induced hyperacetylation of parasite histone, with no or little inhibition of apicidin induced hyperacetylation of host histone, would be considered selective parasitic histone acetyl transferase inhibitors.

In another aspect the present invention provides a method for the treatment of protozoal infections comprising administering to a host suffering from a protozoal infection a therapeutically effective amount of a compound which inhibits histone acetyl transferase. A therapeutically effective amount may be one that is sufficient to inhibit histone acetyl transferase of the causative protozoa.

An example of a compound which has shown histone acetyl transferase inhibiting properties and therefore useful in the treatment of protozoal diseases is represented by the chemical structure (I) below.



(I)

Compound I can be prepared using techniques described in V.I. Saloutin, et al., *J. Fluorine Chemistry*, 56(3):325-334 (1992); K.C. Joshi, et al., *Indian J. Chem.*, 14:1004 (1976); V.Y. Sosnovskykh, et al., *Geterotskikl Soedin*, 6:847-849 (1998); Q.-F. Wang, et al., *Tetrahedron Lett.*, 39(16):2377-2380 (1998); and S. Eguchi, et al., *Heterocycles*, 42(1):333-339 (1996). For example, 0.08mol of ethylenediamine is added to 0.02mol $CF_3C(O)CH_2C(O)$-2-[(4-NO_2)thienyl] in 30mL of glacial acetic acid at RT. The resulting solution is heated to reflux for 2h. The solution is then cooled to RT, poured into 250mL water and extracted with three 50mL aliquots of diethyl ether to remove unreacted beta-diketone. The aqueous layer is neutralized with a 20% NaOH solution and is then extracted with benzene. The 1,4-diazepine base thus obtained is crystallized from benzene or purified by flash chromatography on silica gel.

Active HDA inhibiting compounds include TAN-1746, HC-toxin, chlamydocin, WF-3161, trapoxin A, Cly-2 and analogues thereof. However, analogues of such compounds are good sources of test compounds for screening by the methods of this invention.

Apicidin Ia, Ib, Ic are described in pending applications USSN 08/281,325 filed July 27, 1994 and 08/447,664 filed May 23, 1995. They are produced from a strain of *Fusarium* as disclosed in the above mentioned applications.

Histone acetyl transferase inhibitors are useful as antiprotozoal agents. As such, they can be used in the treatment and prevention of protozoal diseases in human and animals, including poultry. Examples of protozoal diseases against which histone acetyl transferase inhibitors may be used, and their respective causative pathogens, include: 1) amoebiasis *(Dientamoeba* sp., *Entamoeba histolytica)*; 2) giardiasis *(Giardia lamblia)*; 3) malaria *(Plasmodium* species including *P. vivax, P.*

falciparum, P. malariae and P. ovale); 4) leishmaniasis *(Leishmania* species including *L. donovani, L. tropica, L. mexicana, and L. braziliensis)*; 5) trypanosomiasis and Chagas disease *(Trypanosoma* species including *T. brucei, T. theileri, T. rhodesiense, T. gambiense, T. evansi, T. equiperdum, T. equinum, T. congolense, T. vivax* and *T. cruzi)*; 6) toxoplasmosis *(Toxoplasma gondii)*; 7) neosporosis *(Neospora caninum);* 8) babesiosis *(Babesia* sp.*)*; 9) cryptosporidiosis *(Cryptosporidium* sp.*)*; 10) dysentary *(Balantidium coli)*; 11) vaginitis *(Trichomonas* species including *T.vaginitis,* and *T. foetus)*; 12) coccidiosis *(Eimeria* species including *E. tenella, E. necatrix, E. acervulina, E. maxima and E. brunetti, E. mitis, E. bovis, E. melagramatis, and Isospora* sp.*)*; 13) enterohepatitis (*Histomonas gallinarum*), and 14) infections caused by *Anaplasma* sp., *Besnoitia* sp., *Leucocytozoan* sp., *Microsporidia* sp., *Sarcocystis* sp., *Theileria* sp., and *Pneumocystis carinii*.

Histone acetyl transferase inhibitors are preferably used in the treatment or prevention of protozoal infections caused by a member of the sub-phylum Apicomplexans. More preferably histone acetyl transferase inhibitors are preferably used in the treatment or prevention of malaria, toxoplasmosis, and cryptosporidiosis in humans and animals; and in the management of coccidiosis, particularly in poultry, either to treat coccidial infection or to prevent the occurrence of such infection. Further, although not caused by an Apicomplexan, trypanosomiasis may be treated by histone acetyl transferase inhibitors.

In the case that a histone acetyl transferase inhibitor is expected to be administered on a chronic basis, such as in the prevention of coccidiosis in poultry, the histone acetyl transferase inhibitor preferably is selective for protozoal over the host histone acetyl transferase. Long term administration of such a selective inhibitor would minimize adverse effects to the host due to histone acetyl transferase inhibition.

Two specific examples of using histone acetyl transferase inhibitors to prevent the establishment of parasitic infections in humans and animals are 1) the prevention of *Plasmodium* (malaria) infection in humans in endemic areas and 2) the prevention of coccidiosis in poultry by administering the compound continuously in the feed or drinking water. Malaria is the number one cause of death in the world. The disease is transmitted by mosquitoes in endemic areas and can very rapidly progress to a life threatening infection. Therefore, individuals living in or visiting areas where malaria carrying mosquitoes are present routinely take prophylactic drugs

to prevent infection. The histone acetyl transferase inhibitor would be administered orally or parenterally one or more time(s) a day. The dose would range from about 0.01mg/kg to about 100mg/kg. The compound could be administered for the entire period during which the patient or animal is at risk of acquiring a parasitic infection.

Coccidiosis is a disease that can occur in animals and is caused by several genera of coccidia. The most economically important occurrence of coccidiosis is the disease in poultry. Coccidiosis in poultry is caused by protozoan parasites of the genus *Eimeria*. The disease can spread quite rapidly throughout flocks of birds via contaminated feces. The parasites destroy gut tissue, thereby impairing nutrient absorption. An outbreak of coccidiosis in a poultry house can cause such dramatic economic losses for poultry producers that it has become standard practice to use anticoccidial agents prophylactically in the feed. A histone acetyl transferase inhibitor would be administered in the feed or drinking water for, a portion of, or the entire life of the birds. The dose would range from about 0.1ppm to about 500ppm in the feed or water.

For treatment of established parasitic infections in humans or animals, the histone acetyl transferase inhibitor could be administered orally or parenterally once the infection is suspected or diagnosed. The treatment period would vary according to the specific parasitic disease and the severity of the infection. In general the treatment would be continued until the parasites were eradicated and/or the symptoms of the disease were resolved. Two specific examples are the treatment of a 1) *Cryptosporidium parvum* infection in an animal or human and treatment of acute *Plasmodium falciparum* malaria in humans. *Cryptosporidium parvum* is a protozoan parasite that infects and destroys cells lining the intestinal tract of humans and animals. The infection establishes quite rapidly and has acute effects on the patient. In the case of humans, patients get severe dysentery for a period of 5-7 days. In immune compromised patients *C. parvum* infections can persist and can be life threatening. In animals *C. parvum* infection is the number one cause of death in young dairy calves. A *C. parvum* infection can be easily diagnosed by symptoms and examination of a stool sample. Once the disease is suspected and/or diagnosed treatment with a histone acetyl transferase inhibitor can be initiated. The dose would vary from about 0.01mg/kg to about 500mg/kg. Treatments would be one or more time(s) a day, orally or parenterally until the infection is eliminated. Routinely this dosing period would be 1-3 weeks.

P. falciparum causes acute life threatening malarial infections in humans. The infection if left untreated can quite often result in death of the patient. A malaria infection can be easily diagnosed by symptoms and/or examination of a blood sample from the patient. Treatment would be initiated following diagnosis. A histone acetyl transferase inhibitor would be administered one or more time(s) a day, orally or parenterally, until the infection was eliminated. The dose would range from about 0.01mg/kg to about 200mg/kg.

Histone acetyl transferase inhibitors may be administered to a host in need of treatment in a manner similar to that used for other antiprotozoal agents; for example, they may be administered parenterally, orally, topically, or rectally. The dosage to be administered will vary according to the particular compound used, the infectious organism involved, the particular host, the severity of the disease, physical condition of the host, and the selected route of administration; the appropriate dosage can be readily determined by a person skilled in the art. For the treatment of protozoal diseases in human and animals, the dosage may range from about 0.01mg/kg to about 500mg/kg. For prophylactic use in human and animals, the dosage may range from about 0.01mg/kg to about 100mg/kg. For use as an anticoccidial agent, particularly in poultry, the compound is preferably administered in the animals' feed or drinking water. The dosage ranges from about 0.1ppm to about 500ppm.

The compositions of the present invention comprise a histone acetyl transferase inhibitor and an inert carrier. The compositions may be in the form of pharmaceutical compositions for human and veterinary usage, or in the form of feed composition for the control of coccidiosis in poultry. The term "composition" is intended to encompass a product comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product which results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions of one or more of the ingredients. The composition of the present invention thus includes a composition when made by admixing a histone acetyl transferase inhibitor and inert carrier.

The pharmaceutical compositions of the present invention comprise a histone acetyl transferase inhibitor as an active ingredient, and may also contain a pharmaceutically acceptable carrier and optionally other therapeutic ingredients. The compositions include compositions suitable for oral, rectal, topical, and parenteral

(including subcutaneous, intramuscular, and intravenous) administrations, although the most suitable route in any given case will depend on the particular host, and nature and severity of the conditions for which the active ingredient is being administered. The pharmaceutical compositions may be conveniently presented in unit dosage form and prepared by any of the methods well known in the art of pharmacy.

In practical use, a histone acetyl transferase inhibitor can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral or parenteral (including intravenous).

In preparing the compositions for oral dosage form, any of the usual pharmaceutical media may be employed. For example, in the case of oral liquid preparations such as suspensions, elixirs and solutions, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like may be used; or in the case of oral solid preparations such as powders, capsules and tablets, carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents, and the like may be included. Because of their ease of administration, tablets and capsules represent the most advantageous oral dosage unit form in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be coated by standard aqueous or nonaqueous techniques. In addition to the common dosage forms set out above, histone acetyl transferase inhibitors may also be administered by controlled release means and/or delivery devices.

Pharmaceutical compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association the active ingredient with the carrier, which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation. For example, a tablet may be prepared by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing, in a suitable machine, the active ingredient in a free-

flowing form such as powder or granules, optionally mixed with a binder, lubricant, inert diluent, surface active or dispersing agent. Molded tablets may be made by molding in a suitable machine, a mixture of the powdered compound moistened with an inert liquid diluent. Desirably, each tablet contains from about 1mg to about 500mg of the active ingredient and each cachet or capsule contains from about 1mg to about 500mg of the active ingredient.

Pharmaceutical compositions of the present invention suitable for parenteral administration may be prepared as solutions or suspensions of these active compounds in water suitably mixed with a surfactant such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g. glycerol, propylene glycol and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

Suitable topical formulations include transdermal devices, aerosols, creams, ointments, lotions, dusting powders, and the like. These formulations may be prepared via conventional methods containing the active ingredient. To illustrate, a cream or ointment is prepared by mixing sufficient quantities of hydrophilic material and water, containing from about 5-10% by weight of the compound, in sufficient quantities to produce a cream or ointment having the desired consistency.

Pharmaceutical compositions suitable for rectal administration wherein the carrier is a solid are most preferably presented as unit dose suppositories. Suitable carriers include cocoa butter and other materials commonly used in the art, and the suppositories may be conveniently formed by admixture of the combination with the softened or melted carrier(s) followed by chilling and shaping moulds.

It should be understood that in addition to the aforementioned carrier ingredients the pharmaceutical formulations described above may include, as appropriate, one or more additional carrier ingredients such as diluents, buffers,

flavoring agents, binders, surface-active agents, thickeners, lubricants, preservatives (including anti-oxidants) and the like, and substances included for the purpose of rendering the formulation isotonic with the blood of the intended recipient.

For use in the management of coccidiosis in poultry, a histone acetyl transferase inhibitor may be conveniently administered as a component of a feed composition. Suitable poultry feed composition will typically contain from about 1ppm to about 1000ppm, preferably from about 0.01% to about 0.1% percent, by weight of a histone acetyl transferase inhibitor. The optimum levels will naturally vary with the species of *Eimeria* involved, and can be readily determined by one skilled in the art. Levels of in poultry feed of from about 0.01% to about 0.1% by weight of the diet are especially useful in controlling the pathology associated with *E. tenella*, while the preferred concentration for similar control of intestinal-dwelling species is from about 0.01% to about 0.1% by weight of the diet. Amounts of about 0.01% to about 0.1% by weight are advantageous in reducing the pathogenic effects of both cecal and intestinal coccidiosis.

In the preparation of poultry feed, a histone acetyl transferase inhibitor may be readily dispersed by mechanically mixing the same in finely ground form with the poultry feedstuff, or with an intermediate formulation (premix) that is subsequently blended with other components to prepare the final poultry feedstuff that is fed to the poultry. Typical components of poultry feedstuff include molasses, fermentation residues, corn meal, ground and rolled oats, wheat shorts and middlings, alfalfa, clover and meat scraps, together with mineral supplements such as bone meal, calcium carbonate and vitamins.

Compositions containing a compound of formula I may also be prepared in powder or liquid concentrate form. In accordance with standard veterinary formulation practice, conventional water soluble excipients, such as lactose or sucrose, may be incorporated in the powders to improve their physical properties. Thus particularly suitable powders of this invention comprise about 50 to 100% w/w, and preferably 60% to 80% w/w of the combination together with 0 to 50% w/w and preferably 20% to 40% w/w of conventional veterinary excipients. These powders may either be added to animal feedstuff, for example by way of an intermediate premix, or diluted in animal drinking water.

Liquid concentrates of this invention suitably contain a water-soluble compound combination and may optionally include a veterinarily acceptable water miscible solvent, for example polyethylene glycol, propylene glycol, glycerol, glycerol

formal or such a solvent mixed with up to 30% v/v of ethanol. The liquid concentrates may be administered to the drinking water of animals, particularly poultry.

The following non-limiting examples are provided to illustrate the invention.

In the following Examples, anti-histone H4 sera were prepared according to B.M. Turner, et al., *FEBS Lett.*, 253, 141-145 (1989). Apicidin was prepared according to the procedure described in U.S. Patent No. 5,620,953. All temperatures expressed herein are in °C unless specifically stated otherwise. All percentages expressed herein are in percent by weight unless specifically stated otherwise.

EXAMPLE 1

ELISA test to detect inhibition of Apicidin induced hyperacetylation

Madin Derby Chick Kidney (MDBK) cells were seeded at 25,000cells/mL in MEM (Gibco) with the following additions: i) 10% fetal calf serum (FCS), ii) 1mM Sodium Pyruvate, iii) 10mM HEPES, iv) 0.1mM MEM non-essential Amino Acids Solution, v) 100units/ml Penicillin, and vi) 100µg/ml Streptomycin. The cells were seeded into 96 well plates at 100µL per well. Freshly prepared *E. tenella* sporozoites (20,000/well) were added to the host cells and after 3h the infected cells were washed to remove extracellular sporozoites. Fresh media as described above but with 2% heat-inactivated FCS (instead of 10%) added. The infected cultures were incubated at 41°C for 48h.

After 48 hours, the cells were treated with a mixture of 250ng/mL Apicidin and the test compound in a suitable solvent such as DMSO. The resulting treated cells were then incubated at 41°C for 5 hours, and washed with PBS (phosphate buffered saline) 3 times. The washed cells were lysed by 3 successive rounds of freeze thawing followed by being blocked with 5% fat free dried milk in PBS with 0.5% Tween® 20 (polyoxyethylene (20) sorbitan monolaurate) at 200µL/well for 1 hour at 37°C with shaking. The blocking solution was removed and primary antibodies added (anti-hyperacetylated Histone H4 diluted 1:250 in 1% milk/PBS with 0.5% Tween® 20 at 100µL/well) for 1 hour at 37°C with shaking. The wells were then washed 6 times with a solution of 0.5% Tween® 20 in PBS and a secondary antibody added.

The secondary antibody was donkey anti-rabbit conjugated to horseradish peroxidase, diluted at 1:10,000 in 1% milk in PBS containing 0.5% Tween® 20. 100µL of the diluted secondary antibody was added per well followed by incubation for 1 hour at 37°C with shaking. Cells were then washed 3 times with a 0.5% Tween® 20 solution in PBS, followed by 3 more washings with PBS alone. To each plate was added 150µL of a K-blue substrate solution available from Elisa Technologies. The plates were then incubated for 30 minutes at room temperature. The optical density at 570nm was determined, which provided a measure of acetylation.

EXAMPLE 2

Histone Acetyl Transferase Inhibition Assay

HAT assay on unsporulated oocysts

Preparation of the lysate:

E. tenella oocysts. Approximately 2 x 10^9 *E. tenella* oocysts were suspended in 5mL of 50 mM HEPES pH 7.4. Next, 4mL of an equal mixture (vol/vol) of 4.0mm and 1.0mm glass beads was added. The resulting glass bead/oocyst mixture was then shaken for 20 minutes to cause disruption of the oocysts. The efficiency of breakage was checked microscopically. The resulting homogenate was separated from the glass beads and centrifuged at 3000 x g for 10min. The pellet was then mixed with 20mL 50mM Tris (Tris(hydroxymethyl)aminomethane) pH 8, 0.25M sucrose, 0.5M NaCl, 1mM dithiothreitol (DTT), 15mM $MgCl_2$, and 0.1mM phenylmethylsulfonyl fluoride (PMSF). The resulting mixture was stirred for 30min on ice, centrifuged at 100,000 x g for 1hr at 4°C and the supernatant dialyzed overnight at 4°C against 50mM Tris pH 8, 1mM DTT, 15mM $MgCl_2$, and 0.1mM PMSF. The dialysate was made 10% in glycerol, aliquoted, and was then frozen at –80°C or used immediately.

The assay:

About 10µL of extract containing HAT was mixed with Buffer A, which was the combination of i) 50mM Tris pH 8, ii) 0.25M sucrose, iii) 1mM DTT, iv) 15mM $MgCl_2$, and v) 0.1mM PMSF to a volume of 40µL. To the resulting mixture was added 5µL of 200mM butyric acid, followed by adding 25µg calf thymus histones (5µL of 5mg/ml histones in Buffer A). Next, the test compound was added

at a known concentration in a suitable solvent such as DMSO. A dilute label (^{3}H acetyl CoA) was made at a dilution of 1:10 in buffer A to a level of 0.01μCi/μL. Reaction was started by adding 5μL of ^{3}H acetyl CoA (50nCi, 3Ci/mmol). After 10min at 30°C the resulting mixture was then spotted onto Whatman™ P81 filters. The filters were washed with 100mL 50mM NaH_2CO_3/Na_2HCO_3 pH 9.2 for 30 minutes, followed by washing sequentially with acetone then 1:2 v/v chloroform methanol. Then, they were dried and radioactivity was measured by liquid scintillation counting.

Alternatively, after completion of the incubation, the sample was mixed with an equal volume of Tris-Glycine SDS Sample buffer available from Novex and analyzed by SDS gel electrophoresis followed by detection of labeled histones by fluorography or autoradiography, as known in the art. Inhibition of the histone acetyl transferase was measured by a reduction in the number of counts per minute associated with histones either trapped on the filter or in a protein of appropriate molecular weight.

Gel electrophoresis:

Acid urea triton (AUT) polyacrylamide gels were performed according to the methods of Alfageme, et al., *J. Biol. Chem.* 249, 3729, (1974) with modifications as described in Lennox and Cohen, *Methods in Enzymology*, 170, 532-549 (1989). For optimal resolution of MDBK cell and *E. tenella* histones, the separation gel contained i) 7.5M urea, ii) 12% acrylamide, iii) 0.38% Triton® X 100 available from Aldrich Chemical Company, Milwaukee, Wisconsin, iv) 0.8% bis acrylamide, and v) 0.87M acetic acid. The loading gels contained i) 7.5M urea, ii) 0.37% Triton® X 100, iii) 6% acrylamide, iv) 0.04% bis acrylamide and v) 0.87M acetic acid. Gels were run in 1M acetic acid. Gels were pre-electrophoresed at 350V for 1 hour and run at 450V for 3.5 hours. Gels were either stained with Coomassie® Brilliant Blue R available from Aldrich Chemical Company, Milwaukee, Wisconsin in 7% acetic acid, 20% methanol and then destained in 7% acetic acid and 20% methanol, or treated with Enlightening (from New England Nuclear), dried and radiolabel detected by fluorography.

WHAT IS CLAIMED IS:

1. A method for identifying compounds having antiprotozoal activity, said method comprising the steps of:

contacting a first amount of an histone acetyl transferase with (i) a second amount of an identifiably labeled compound that interacts with histone acetyl transferases; and (ii) a third amount of a test compound or composition; and

quantitating said labeled compound effective to determine a level of histone acetyl transferase activity, wherein said level is induced by said third amount.

2. The method of Claim 1 wherein said labeled compound binds to histone acetyl transferase.

3. The method of Claim 1 wherein said labeled compound is a substrate of histone acetyl transferase.

4. The method of Claim 1 wherein said histone acetyl transferase is a protozoal histone acetyl transferase.

5. The method of Claim 4, further comprising the steps of:

contacting a fourth amount of a host histone acetyl transferase with (i) a fifth amount of said identifiably labeled compound; and (ii) a sixth amount of said test compound or composition; and

quantitating said labeled compound effective to determine a level of host histone acetyl transferase activity, wherein said level is induced by said sixth amount.

6. A method for identifying compounds having antiprotozoal activity, said method comprising the steps of:

contacting histone with i) a first amount of an histone acetyl transferase, (ii) a second amount of a histone deacetylase, (iii) a third amount of a histone deacetylase inhibitor, (iv) a fourth amount of a test compound or composition, and (iii) a fifth amount of an antibody that interacts with acetylated histone; and

quantitating said antibody effective to determine a level of acetylated histone, wherein said level is induced by said fourth amount.

7. The method according to Claim 6, further including the prior step of:

determining a hyperacetylation level against which is compared said level of acetylated histone.

8. The method of Claim 6 wherein said histone acetyl transferase is a protozoal histone acetyl transferase.

9. The method of Claim 8, further comprising the steps of:

contacting histone with i) a sixth amount of a host histone acetyl transferase, (ii) a seventh amount of a histone deacetylase, (iii) an eighth amount of a histone deacetylase inhibitor, (iv) a ninth amount of a test compound or composition, and (iii) a tenth amount of an antibody that interacts with acetylated histone; and

quantitating said antibody effective to determine a level of acetylated histone, wherein said level is induced by said ninth amount.

10. A method for identifying compounds having histone acetyl transferase inhibiting activity, said method comprising the steps of:

determining a reference histone hyperacetylation level in a histone/histone acetyl transferase/histone deacetylase/histone deacetylase inhibitor system;

determining a test histone acetylation level in a histone/histone acetyl transferase/histone deacetylase/histone deacetylase inhibitor/test composition system; and

comparing the test level to the reference level.

11. The method of Claim 10, further including a step of:

determining a basal histone acetylation level in a histone/histone acetyl transferase/histone deacetylase system; and

wherein said comparing step includes comparing the test level to the basal level.

12. A method for identifying compounds having histone acetyl transferase inhibiting activity, said method comprising the steps of:

determining a first histone acetylation level in a histone/histone acetyl transferase/histone deacetylase/test compound system at a first concentration of test compound;

determining a second histone acetylation level in a histone/histone acetyl transferase/histone deacetylase/test compound system at a second concentration of test compound; and

comparing said first and second histone acetylation levels.

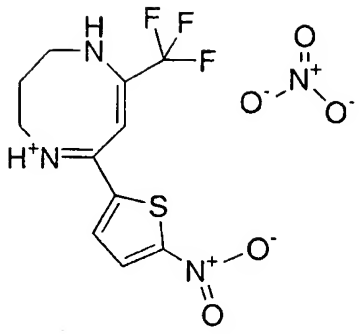
13. A method for the treatment or prevention of a protozoal disease comprising a step of administering to a host a therapeutically or prophylactically effective amount of a compound which inhibits histone acetyl transferase of the disease protozoa.

14. The method of Claim 13 wherein said compound inhibits protozoal histone acetyl transferase to a greater extent than host histone acetyl transferase.

15. The method of Claim 13 wherein said protozoal disease is caused by a protozoan belonging to the sub-phylum Apicomplexans.

16. The method of Claim 13 wherein said protozoal disease is coccidiosis, malaria, cryptosporidiosis or toxoplasmosis.

17. The method of Claim 13 wherein said compound is represented by chemical structure (I):



(I).

18. A method for identifying compounds having antiprotozoal activity comprising:

(a) contacting an intact host or protozoal cell with a test compound;

(b) disrupting said cell to obtain histones; and

(c) determining the level of histone acetylation.

19. The method of Claim 18 wherein said test compound is a natural product extract.

20. The method of Claim 18 wherein the level of histone acetylation is determined using acid urea triton gel electrophoresis.

21. A composition useful for the prevention or treatment of protozoal diseases which comprises an inert carrier and an effective amount of a histone acetyl transferase inhibitor.

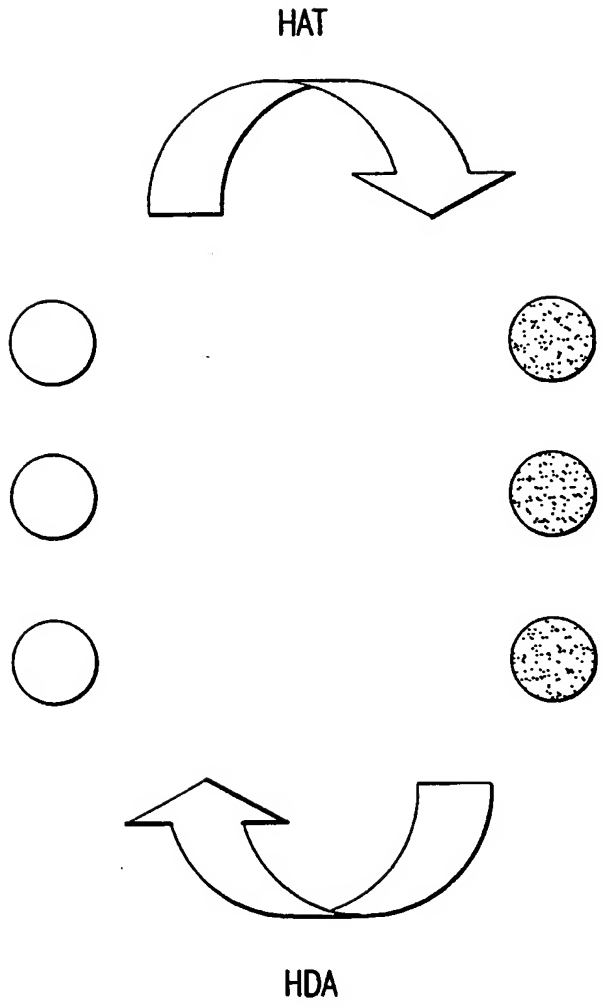


FIG.1a

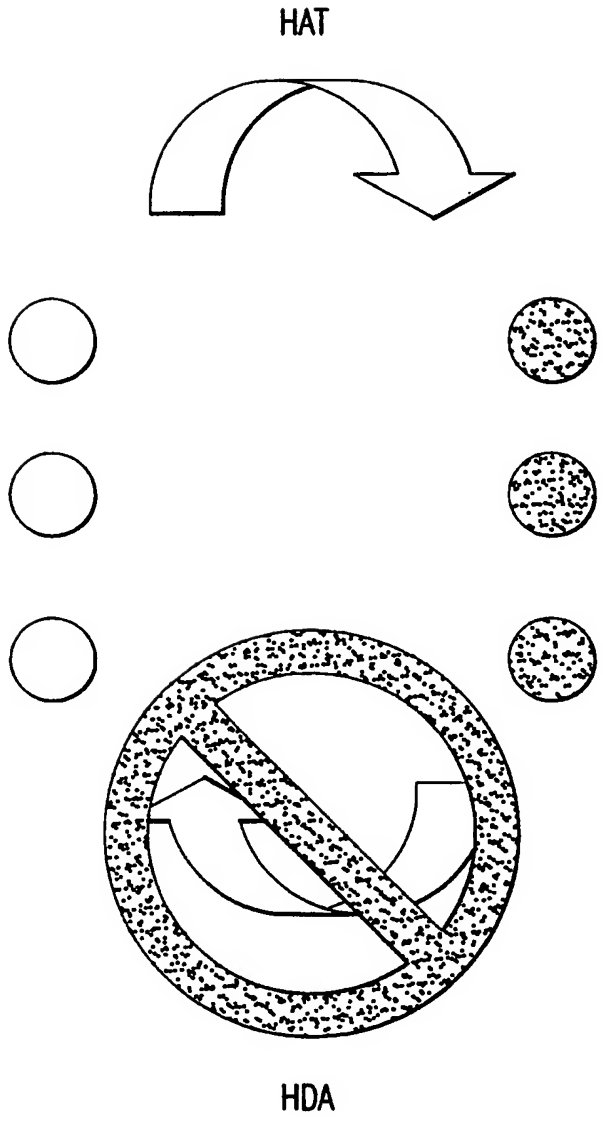


FIG.1b

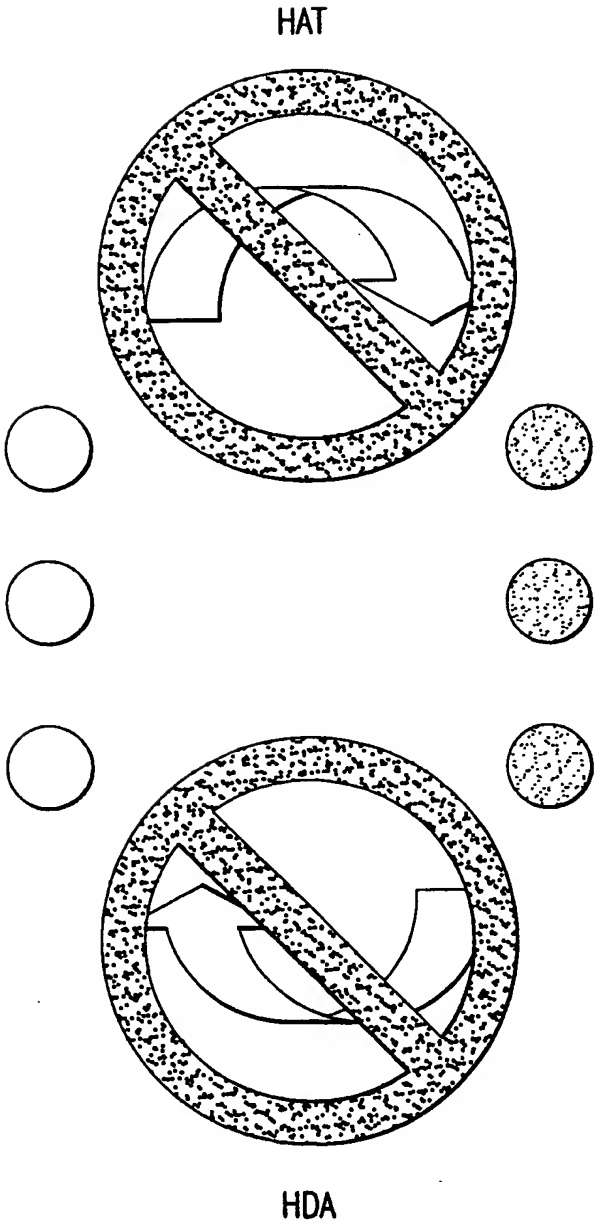


FIG.1c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27337

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/34; C12N 9/14, 15/09; A01N 43/02

US CL :435/18, 69.2, 195, 252.3, 7.22; 514/183, 449, 895

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/18, 69.2, 195, 252.3, 7.22; 514/183, 449, 895

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 6,110,677 A (DULSKI et al) 29 August 2000, entire document.	1-21
A	US 6,068,987 A (DULSKI et al) 30 May 2000, entire document	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 JANUARY 2001	Date of mailing of the international search report 08 MAR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Dorothea Lawrence For PADMA BASKAR Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27337

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27337

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST, HCAPLUS, CAOLD, MEDLINE, USPATFUL, CHEMCATS, WPIDS, BEILSTEIN, MARPAT, REGISTRY, CAPLUS.
search terms: histone, inhibitors, histone actyl transferase, treat, prevent, diazepin? histone deacetylsa? malari? or coccidiosis or crytosporidiosis or toxoplasmosi? test copmpound, inhibit? or assay? or determine? or identif? or activat?

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-5, drawn to a method for identifying compounds having antiprotozoal activity.
Group II, claim(s) 6-9, drawn to a different method for identifying compounds having antiprotozoal activity, using an antibody.
Group III, claim(s) 10-12, drawn to another method for identifying compounds having antiprotozoal activity, using a reference histone hyperacetylation level to test level.
Group IV, claim(s) 13-17, drawn to a method for the treatment or prevention of a protozoal disease using a compound that inhibits histone acetyl transferase.
Group V, claim(s) 18-20, drawn to a method for identifying compounds having antiprotozoal activity, using natural product extract.
Group VI, claim(s) 21 drawn to a composition for the treatment or prevention of a protozoal disease which comprises an inert carrier and an effective amount of histone acetyl transferase inhibitor.
The inventions listed as Groups I- VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The Groups I-V inventions are drawn to different methods having different goals, method steps and starting materials, which do not require each other for their practice and do not share the same or a corresponding technical feature. Group VI is drawn to a composition useful for the prevention or treatment of protozoal disease. Note that PCT Rule 13 does not provide for multiple products or methods within a single application. Since the special technical feature of the Group I invention is not present in the Group II-VI claims, and the special technical features of the Group II-VI inventions are not present in the Group I claims, unity of invention is lacking.